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13. ABSTRACT (Maximum 200 Words)

Introduction: Lymphedema is an abnormal swelling, in which lymph production exceeds drainage capabilities. This occurs as a result of lymphatic vessel destruction during the removal of lymph nodes or subsequent radiation therapy in breast cancer treatment. Management of lymphedema remains a clinical problem. In adult lymphangiogenesis, VEGF-C has been shown to be a specific mitogen for lymphatic endothelial cells (LEC) via the VEGF-3 receptor. Ang-2 has recently been shown to be required for proper lymphatic development via the Tie 2 receptor. Objective: In our model we incorporated into alginate gels, Ang-2 and VEGF-C to promote lymphoangiogenesis by stimulating LEC proliferation and migration. Methods: Sterile alginate gels with Ang-2 ($2\mu g/ml$) and VEGF-C ($200ng/ml$) were tested *in vitro* for proliferation and migration. Results: By adding Ang-2 to the VEGF-C alginate gels, LEC proliferation and migration increased, when compared to VEGF-C alginate gels. Conclusions: Our in vitro results demonstrate that alginate gels are an effective delivery system of Ang-2 and VEGF-C, in which new tubular structures are formed resembling lymphatic vessels. Further studies are required to evaluate these new tubular structures and their capability in restoring lymphatic function in lymphedema animal models *in vivo*.

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Table of Contents

Introduction:

Breast carcinoma continues to be the most frequently occurring carcinoma in women. Presently, approximately one in every eight women will develop the disease.[1] Great strides have been made in the treatment of breast carcinoma that have reduced the risk of recurrence and improved survival rates.

The removal of axillary lymph nodes has been an integral part of breast carcinoma treatment since the end of the last century.[2] Axillary lymph nodes are removed to provide accurate information for staging, to accomplish local control, and for prognosis in order to plan adjunctive, systemic therapy. The status of the axillary lymph nodes remains the single most important predictor of survival.[3] Therefore, axillary treatment surgically and/or with radiotherapy is still an essential component in the management of most patients with invasive breast carcinoma.[4-6]

Chronic edema of the arm, commonly called Lymphedema or post-mastectomy edema (PME), was described first as a side effect of mastectomy operations by Halsted in 1921.[7] Although there has been a trend toward more conservative surgery and greater use of radiotherapy, PME remains a common iatrogenic problem (*Figure1*).

Figure 1: Photograph of Secondary Lymphedema due to Breast Cancer treatment. Lymph stagnation within the left arm's adipose tissue and subsequent swelling (edema).

Edema represents an increase in interstitial fluid volume sufficient to manifest with swelling. Any edema, whatever the underlying cause, is due to an imbalance between capillary filtration and lymph drainage.[8] Most examples of limb edema are caused by an increase in capillary filtration, overwhelming lymph drainage capacity. Lymphedema, however, strictly occurs when swelling is due to a failure of lymph drainage in circumstances in which capillary filtration is not increased.

The lymphatic system is a one-way drainage route designed to rid the "tissues" of unwanted material and excess fluid. It therefore represents a waste route and overflow pipe, with its essential function being to return to the blood vascular compartment protein, colloids, and particulate matter too large to reenter the blood compartment directly [9] (*Figure 2*). Two types of lymphatic vessels exist: First, the smaller initial lymphatic, which includes the smallest lymphatic capillary and the larger pre-collector vessel, and second, the collecting lymphatic vessel into which the pre-collectors drain.[10]

The collecting lymphatics are the main limb lymphatic vessels that provide the afferent flow to the lymph nodes. They behave like a series of smooth muscle hearts that are responsible for the propulsion of lymph centripetally.[11] Intrinsic pumping of collecting

4

vessels is essential motor for lymph propulsion. For initial lymphatics, however, flow of interstitial fluid and macromolecules is caused by intermittent changes in hydrostatic and oncotic pressures locally.[12] Deformation or movement of the tissues by surface pressure or underlying muscle contractions and by other contractile structures, such as arterioles,
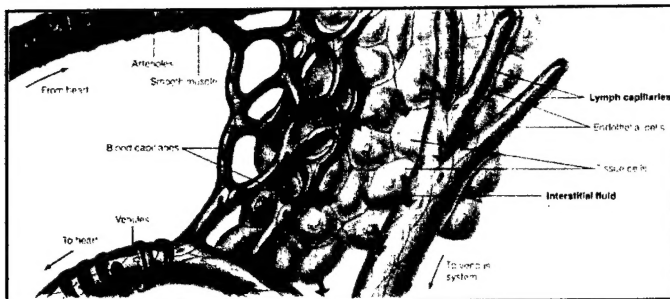

Figure 2:
Schematic representation of lymphatic vessel, arteriole and venule in the interstitial space. Proteins unable to be incorporated into the lymphatic circulation accumulate in the adipose tissue causing edema.

causes expansion or compression of the initial lymphatics. The compression forces lymph along initial lymphatics. Valves in both, initial and collecting lymphatics ensure that flow is unidirectional. Lymphedema arises when an intrinsic fault develops within the lymph-conducting pathways (primary lymphedema) or when damage occurs from one or more factors originating outside the lymphatic system, such as surgical removal of lymph nodes (secondary lymphedema).

Lymphedema therefore, is a disease characterized by abnormal collections of fluid and proteins within the interstitial space. Treatment options for this debilitating condition have included drug therapy, physical therapy, and surgical approaches that have yielded limited success. Unfortunately, for lymphedema today, treatment options are all palliative, for there is **no effective treatment** that could offer a permanent cure to this disease.

Secondary Lymphedema resulting after iatrogenic, surgical disruption of the lymphatic vessels in breast cancer surgery, has continued to be neglected in the U.S. in spite of the fact that it has now become an acceptable diagnosis (ICD9-457.0).[13] Swelling of the upper limb, constitutes the most invalidating complication of breast carcinoma treatment. Untreated, upper extremity lymphedema predisposes women to the development of severe acute or chronic infections with limitations on functioning and serious disturbances in a patient's quality of life. Psychological distress (disfigurement), depression and social inhibition.[14-15]

It is estimated that 15-20% of the 2 million breast cancer survivors, are living currently with post-treatment lymphedema.[16-17] The swollen arm, which can be as much as twice the normal size, is disfiguring and commonly causes functional impairment, psychosocial maladjustment and psychological morbidity.[18] Added to the physical symptoms that patients must cope with, is the pain caused unintentionally by clinicians that, interested in carcinoma recurrence, trivialize the non-lethal nature of lymphedema.

Body:

Vascular Endothelium Growth Factor (VEGF) is produced by many different cell types both in tissue culture and in vivo. It binds to plasma membrane receptors on endothelial cells (EC) only with an extra cellular transmembrane glycoprotein linked to an intracellular tyrosine kinase domain, thus VEGF is an EC specific mitogen.

VEGF-C is a 38 kD homodimeric glycoprotein that is commercially available. This recombinant human VEGF-C is from a DNA sequence encoding the 165 amino acid residue variant of human VEGF expressed in Sf 21 insect cells using a baculovirus expression system. The product is lipolized from a sterile-filtered solution in 30% acotonitrile plus 0.1% TFA containing 50µg of cytokine. **VEGF-C** is of particular interest because it is a specific mitogen for **lymphatic endothelium** in adult tissues.

Angiopoietin-2 is a ligand for the endothelial Tie-2 receptor tyrosine kinase, it has a dual function in the processes of postnatal angiogenesis and vascular remodeling. Also **Ang-2** signals are required for the proper development and function of **lymphatic vessels.** Recent data also suggest that the roles of Ang-2 and VGEF-C in the lymphatic vessel development are analogous too the roles of Ang-1 and VEGF in blood vessel development. Thus, Ang-2 is the first endothelial cell-specific growth factor demonstrated to function in vessel formation or regression depending on the tissue context.[19] In combination with VEGF-C, Ang-2 potentiates its effect.

Tissue engineering is an interdisciplinary field that incorporates principles of engineering and polymer chemistry into the biological sciences, in efforts to develop biological substitutes for failed tissues and organs. It is a new and rapidly expanding field, in which the techniques are being developed for culturing or promoting regeneration of a variety of tissues, both in vitro and in vivo using polymer "scaffolds" to support tissue growth.[20]

Unlike the solid polymer systems currently used to create a cell-polymer construct, a liquid support matrix that polymerizes to a gel would have the potential for injectable delivery, which would be much less invasive than open implantation. **Alginate hydrogels** have been increasingly important in biotechnology applications, such as tissue engineering, artificial organs and as drug carriers.[21] Delivery rate can be predetermined and its local, rather than systemic administration be of great advantage (Alginates are approved by Food and Drug Administration for human use).

Alginate refers to a family of polyanionic copolymers derived from brown sea algae and comprising 1,4-linked β-D-manuronic (M) and α-L-glucoronic (G) acid residues in varying proportions. The fact that G and M are C5 epimers results in a switch-over of the monomer chair conformation, giving rise to all four possible glycosidic linkages and at the molecular level, large effects like cavity formations between G residues are observed. It has been shown that the occurrence of G and M within the alginate molecule is block-wise and not random.[22,23]

There is a direct dependence between the mechanical strength of an alginate gel and the porosity of the gel network. When gels are made from alginate rich in glucoronic (G) acid residues, higher moduli are obtained compared to gels made from alginates less enriched in G residues, and also higher diffusion rates. *(Figure 3)*. Gelling properties of alginate are a function of the M/G composition and the sequential structure of M and G along the alginate chain (*Figure 3*).
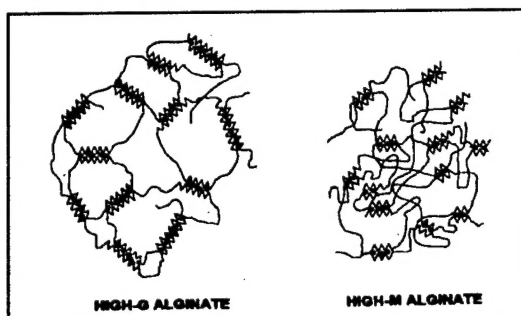

In general terms, an increase in G content (measured as $G_{n>1}$) as well as in molecular weight will give a stronger gel. In contrast to most gelling polysaccharides, alginate gels have the particular feature of being "cold setting"'. This implies that the setting of alginate gel is more-or-less independent of temperature.

Figure 3

Alginate gel incorporation: Essentially there are two main methods for the preparation of alginate gels: The dialysis/diffusion method and internal geleation method. In the dialysis/diffusion method (diffusion setting) gelling ions are allowed to diffuse into the alginate solution. In our study we will incorporated sterile G (50%) and M (50%) copolymers of alginate to a solution with Potassium Phosphate and Sodium Chloride (0.1 M K_2HPO_4 and 0.135 M NaCl, pH of 7.4), which was previously sterilized by autoclave. A 1.0% Sodium Alginate solution will then be mixed with 0.2 gm of $CaSO_4$ per milliliter, this solution will be placed in ice prior to extrusion. Because of all the different characteristics previously mentioned we decided to produce an alginate gel that would have the best properties of both, the G and M copolymers. Thus providing a gel that would be highly porous and relatively strong. Previously expanded EPC's which have been induce to differentiate into LEC by the growth factors, VEGF-C and Ang-2, will be incorporated into this gel for injection.

Animal Model for LEC harvest:

We continue to use the same animal model described last year to harvest the LEC to grow in culture to use in our in vitro studies.

Preliminary in vitro studies were carried out to determine the ideal Ang-2 concentration to be incorporated into the alginate gels. *In vitro* culture with LEC and three different concentrations (as recommended by Regeneron): 0.22 µg/ml, 0.67 µg/ml and 2 µg/ml of Ang-2 reveled that the proliferative and migratory response of the LEC was greater at 2 µg/ml when compared to the other two concentrations.
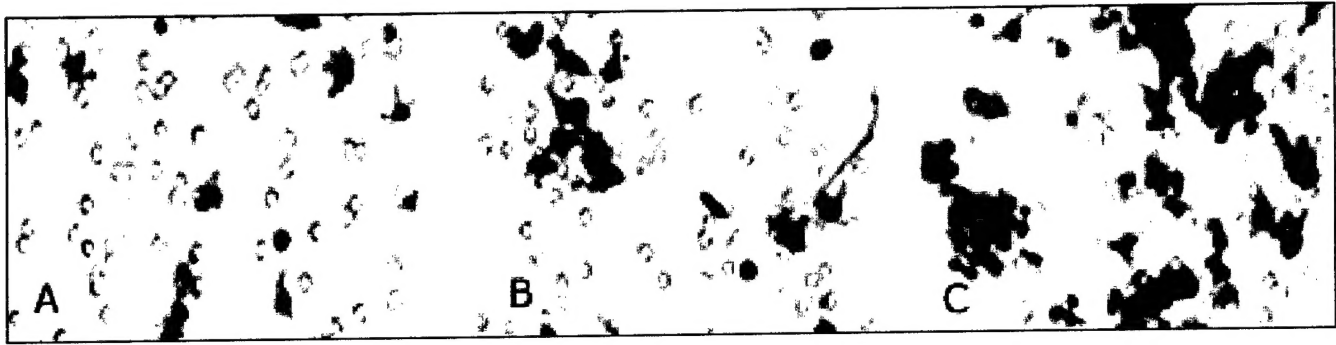
Figure 4. Photograph of in vitro Proliferation and migration Assays of LEC in a Bodin chamber at 7 days. A) Ang-2 concentration of 0.22 µg/ml, B) Ang-2 concentration of 0.67 µg/ml, C) Ang-2 concentration of and 2 µg/ml.

With these initial results we determined that the concentrations of Ang-2 that should be incorporated into alginate gels should be the same as the ones above, and determine if the release of the growth factor from the alginate gel would result in the same or comparable proliferation and migration of LEC.

LEC's were cultivated with Ham's modified F12K medium for EC with ECGS. Cells were serum starved (from 10% to 1%FBS) prior to plating on the petri-dish with the alginate gels. Once again the ideal concentration for Ang-2 was that of 2 µg/ml, where the proliferation and migration of LEC was more profound.

Alginate gels were then prepared to incorporate VEGF-C at a concentration of 200 ng/ml and Ang-2 at a concentration of 2 µg/ml. These gels will now be used *in vivo*, in the mouse tail animal model we have worked on.

Lymphedema animal model: A lymphedema mouse model has recently been described in the literature.[24] This model provides a useful *in vivo* system, in which experimental treatments for lymphedema can be evaluated. We have started to operate on the tail of mice to cauterize the lymphatics and induce lymphedema (*Figure 6, 6a*).

In this model, hairless mice (SKH1/Charles River Laboratories, Pittsfield, NH) weighing between 20 are 30 g will be used. The hairless mice offer the advantage that lymphedema development and subsequent tail diameter measurements are easier to follow. Anesthesia and Analgesia: The animals are anesthetized by placing them inside an induction chamber with 1.5% isoflurane and 100% Oxigen (JA Webster Inc., Sterling, MA). Once the animals are anesthetized they are placed in a prone position and the head placed inside a conical mask to maintain anesthesia (0.8% Isoflurane and 100% Oxigen). Analgesia is maintained post-op and for the next 7 consecutive days by daily subcutaneous (SC) injections of 3.3 mg/Kg butorphanol.

8

Prior to start the surgical procedure, 0.10 ml of 5% blue dextran 2M (Sigma, St. Louis, MO) in saline is injected SC into the tip of the tail in order to identify the lymphatic vessels. Then, the operative site at the base of the tail is cleansed with 70% ethanol and povidine/iodine, and a circumferential incision is made through the dermis to sever the superficial lymphatic network. The three deep lymphatic vessels located in the lateral and ventral aspects of the tail are isolated and destroyed by electrical cauterization. Finally, low cauterization is applied too the edges of the circumferential wound, which results in a 2-4 mm gap between the skin edges followed by secondary healing at the site (Figure 6).

Figure 6 .
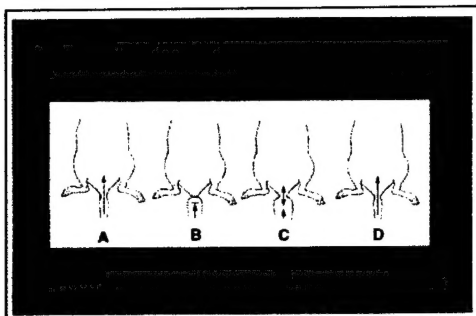
Schematic of Lymphatic tail model.

A) Normal lymphatic flow.
B) Obliteration of tail lymphatics with subsequent lymphedema formation.
C) Lymphangiogenesis by transplantation (Injection) of Alginate gel with VEGF-C and Ang-2.
D) Restoration of normal lymphatic flow with subsequent lymphedema reabsorption.

Once the lymphedema of the tail has been developed (3,7,14 and 28 days), the transplantation (SC injection) of the alginate gel containing the growth factors, VEGF-C and Ang-2 will be performed in an attempt to induce lymphatic vessel proliferation and regeneration of the lymphatic network previously destroyed. Subsequent Lymphocintography studies will confirm if lymphatic flow has been restored.

Ang-2 & VEGF-C Alginate gels Transplantation Animal Model. All procedures will be performed in accordance with the Animal Care Act and Use Committee. Hairless mice (Charles River Laboratories) age 8-10 weeks and weighing 20-30 g will be anesthetized with 160 mg/kg pentobarbital IP. for operative transplant (injection) of the lymphedematous tails.
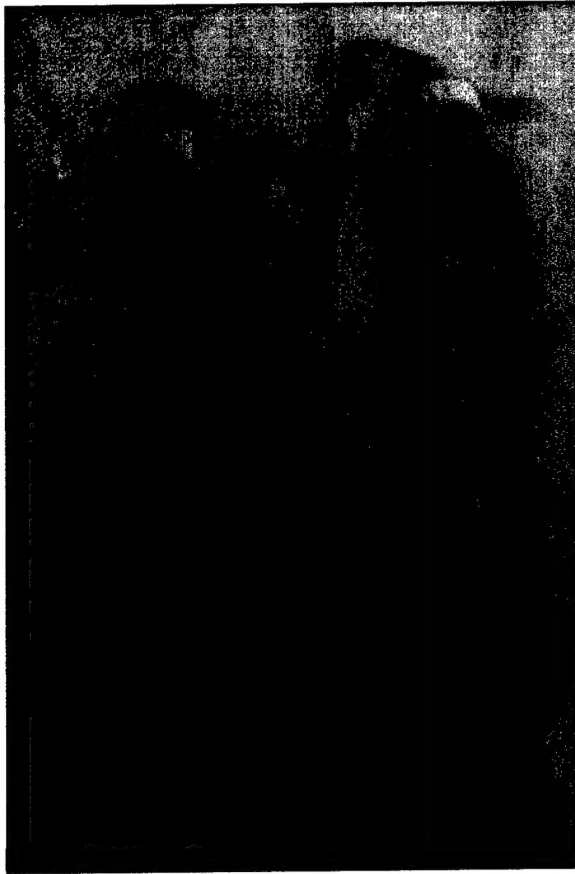
9

Figure 6a.

Photograph of Lymphedematous Tail in the mouse (A), at 2 weeks. The increase in diameter of the tail is more profound at a later time point 4 weeks.
When compared side-by-side to a normal (B) tail of a mouse, the Lymphedematos tail is also evident.
Alginate gels with Ang-2 & VEGF-C will be injected at different time intervals (3, 7, 14, and 28) to evaluate lymphatic regeneration and restoration of lymphatic flow.

Last Phase of Research: *In Vivo* model.

Physiological Assessment of Transplanted Animals. At 3,7,14 and 28 days after transplantation, Lymphocintography studies will be conducted to assess lymphatic regeneration. Immediately before sacrifice, mice will be injected with an overdose of pentobarbital.

Histologic Assessment of Transplanted Animals. Tissue sections from the base of the Lymphedematous and healthy tails will be performed. Tails will be harvested on days 3, 7, 14, and 28 after transplantation. For immunohistochemistry, tissues will be embedded in OCT compound (Miles Scientific, Elkhardt, IN) and snap frozen in liquid nitrogen. Frozen sections of 6-μm thickness will be mounted on silane-coated glass slides, air-dried for 1 h, and counterstained with biotinylated antibodies to UEA-1, mouse and human CD31 (PECAM-1; Dako), and conjugated with FITC-streptavidin (Caltag), as previously described. Before direct immunofluorescent examination, slides will be covered with Vectashield mounting medium for fluorescence microscopy (Vector Laboratories). The extent of lymphangiogenesis will be assessed by measuring lymphatic

density in light microscopic sections of tails retrieved from lymphedematous mousetails. Sections will be stained for LYVE-1 to identify LECs.. At each time point, 20 fields (×40 magnification) will be counted for each of 3 animals.

Statistical Analysis. All data will be presented as mean ± SEM. Inter-group comparisons will be performed by paired Student's t test or ANOVA. The comparative incidence of lymphatic regeneration by decreased lymphedema will be evaluated. Probability values of $P < 0.05$ will be interpreted to denote statistical significance.

Key Research Accomplishments:

Our Specific aims for the second year of this award were successfully completed.

- We were able to incorporate into our previous porous, biodegradable alginate gel with VEGF-C a second growth factor, Ang-2 to serve as a receptor agonist, thus enhance the VEGF-C mitogenic effect in promoting Lymphatic Endothelial Cell proliferation and migration *in vitro*.

- Next Phase we'll induce (surgically) lymphedema of the tail of mice to inject the VEGF-C and Ang-2 alginate gels to evaluate lymphoangiogenesis *in vivo*.

Reportable Outcomes:

- Rat Thoracic Duct Cell line maintained.

- Abstract submitted to the International Meeting on Experimental Biology 2003, San Diego, California. April 11-15, 2003 and was accepted for Oral presentation.

- Abstract printed in the FASEB Journal. ABSTRACTS / Part II (Abstracts A456.1-A886.2) Volume 17, No.5, March 17, 2003.

- We were invited by Dr. Stanley G. Rokson, Chief editor of the Lymphatic Research and Biology Journal to submit a manuscript this fall. Manuscript in progress.

Conclusions:

A biodegradable Alginate gel, may be an effective delivery system for sustained slow release of VEGF-C and Ang-2 to promote lymphangiogenesis in those instances where the integrity of the VEGF-3 and Tie-2 receptor of the LEC are intact. We intend to test these alginate gels in a lymphedema animal model (mouse tail) to evaluate lymphoangiogenesis *in vivo*.

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Appendices:

- Copy of Abstract publication in the FASEB Journal from the Oral Presentation at the Experimental Biology 2003 Meeting.

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ABSTRACTS
PART II

Abstracts A456.1–A886.2

ELISA. We then examined the role of TRAIL. The expression of the four TRAIL receptors was examined by Western blot and was not changed in HMEC plated on PDL compared with those plated on OPN. An anti-TRAIL monoclonal antibody immunoprecipitated OPG in both the lysates and media of serum deprived HMEC plated on OPN. In HMEC plated on PDL in the absence of serum the addition of recombinant TRAIL enhanced the percentage of apoptotic cells whereas a neutralizing antibody against TRAIL decreased apoptosis. Therefore the survival of serum deprived HMEC on OPN is regulated by the secretion of OPG and its subsequent interaction with TRAIL.

514.11

Pericyte tubes: A Novel Species of Angiogenic Microvessels in Oncodevelopmental Tissues

Ugur Ozerdem. Cancer Research Center, The Burnham Institute, 10901 North Torrey Pines Rd., La Jolla, California 92037

This study focuses on elucidating the functional role of pericytes in several complementary models of neovascularization in development and neoplasia. Normal and pathological neovascularization were studied in the following mouse models: normal neonatal retinal angiogenesis, bFGF-induced corneal angiogenesis, and tumor angiogenesis (melanoma, lung, breast, prostate and glial tumors). Using the confocal microscope 3D image reconstruction was utilized to identify pericytes. Pericytes were recognized by expression of the NG2, while endothelial cells were identified by expression of the combined markers CD31, flk 1, and CD105. Proliferating cells were identified by BrdU uptake. The anti-angiogenic efficacy of NG2-neutralizing antibody was evaluated in the mouse corneal angiogenesis assay. Results: BrdU-positive pericytes investing BrdU-positive endothelium were identified in nascent angiogenic sprouts. Microvascular segments formed exclusively by pericytes (pericyte tubes/bridges) lacking endothelial lining were documented in all three models. The mean area of corneal neovascularization was 0.1445 mm2 in the NG2 antibody treated group compared to 0.3863 mm2 in the control group (p=0.0039, n=20). Conclusions: Proliferating pericytes are present in very early angiogenic sprouts and can serve as targets for anti-angiogenic therapy. Identification of pericyte lined segments lacking endothelium suggest a novel vessel type that is different from mosaic vessels and vasculogenic mimicry.

514.12

Promoting Lymphangiogenesis In Vitro Utilizing Alginate Gels with Angiopoietin-2 and Vascular Endothelial Cell Growth Factor-C

Mauricio A. Contreras, Sumner A Slavin. Plastic Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, 4 Blackfan circle / Suite 131, Boston, Ma 02115

Lymphedema is an abnormal swelling, in which lymph production exceeds drainage capabilities. This occurs as a result of lymphatic vessel destruction during the removal of lymph nodes or subsequent radiation therapy in cancer treatment, as well as, obstruction of the lymphatic vessels in filarial infection. Management of lymphedema remains a clinical problem. Restoration of the lymph-transporting capacity is the only therapy that would deal directly with the cause of lymphedema. Vascular endothelial Growth Factor (VEGF) and Angiopoietins (Ang) work in complementary and coordinated fashion during the development of the lymphatic vasculature. In adult lymphangiogenesis, VEGF-C has been shown to be a specific mitogen for lymphatic endothelial cells (LEC) via the VEGFR-3 receptor. Ang-2 has recently been shown to be required for proper lymphatic development via the Tie 2 receptor. In our model we incorporated into alginate gels Ang-2 and VEGF-C to promote lymphangiogenesis by stimulating LEC proliferation and migration. Our in vitro results demonstrate that alginate gels are an effective delivery system of Ang-2 and VEGF-C, in which new tubular structures are formed resembling lymphatic vessels. Further studies are required to evaluate these new tubular structures and their capability in restoring lymphatic function in lymphedema animal models in vivo.

VASCULAR RESPONSES (515.1-515.11)

515.1

Progression of Pulmonary Hypertension: Role of Nitric Oxide and Adrenomedullin

Palaniswamy Vijay, Thomas G Sharp, John W Sharp. Indiana University School of Medicine, 545 Barnhill Drive, EH 215, Indianapolis, IN 46202-5112

Pulmonary hypertension (PH) is a progressive disease leading to right heart failure and eventually death. Here we analyzed the impact of adrenomedullin (ADM), nitric oxide (NO) and gender in the development of chemically induced PH in rats.

PH was induced in SD rats (male/female) by monocrotaline (MCT, 60mg/kwt) and the rats were terminated on days 7 and 21. Control rats received solvent alone. Plasma ADM (pg/ml) and NO (μM) levels and lung nitric oxide synthase NOS (pmol/min/mg protein) activities were measured.

The mortality rates were 33% for male and 3% and for female MCT-treated rats. Plasma NO levels and tissue NOS activity increased in all MCT-treated rats. However, NOS activity peaked at day 7 in male MCT-rats(67±8) and by day 21 NOS activity had declined to baseline(34±7). In contrast, in the female MCT-rats, NOS activity increased throughout the study period. There was a three-fold increase in ADM among male MCT-rats but in female MCT-rats the levels increased more than 5-fold by day 21 (1.7±0.6 to 9.5±1.1) after MCT injection. Control values were unchanged.

The endothelial injury caused by MCT resulted in higher NOS activity and thus increased plasma NO levels quickly while ADM responded in a somewhat delayed fashion. The more sustained increases in both ADM and NOS seen in female MCT-rats may alter the long-term progression of the disease resulting in better survival suggesting a role of female hormones in PH progression.

515.2

Poly(ADP-ribose) polymerase (PARP) activation is an early event in angiotensin-induced cardiovascular disorders

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Angiotensin II (ANG II) is a well-established participant in many cardiovascular disorders. Recent work demonstrated that infusion of ANG II at sub-pressor doses induces the formation of 3-nitrotyrosine (a stable biomarker of tissue peroxynitrite formation). Here we investigated the role of PARP, a multifunctional nuclear enzyme in the development of AII-induced endothelial dysfunction in the rat. AII infusion to Sprague-Dawley rats (200 ng/kg/min) for 7 days induced the development of endothelial dysfunction, as assessed by the endothelium-dependent relaxant responses to acetylcholine in the thoracic aorta ex vivo. This endothelial dysfunction was sustained when AII was infused at the same rate for an additional 1 week period. The PARP inhibitor PJ34 (Nature Med 7:108-113, 2001) at a dose of 20 mg/kg/day, starting from Day 1, prevented the development of the endothelial dysfunction. Administration of PJ34 for the second week only, restored normal endothelial function. Immunohistochemical detection of poly(ADP-ribose) confirmed that PJ34, at the dose of 20 mg/kg/day, fully blocks vascular PARP activation. PARP activation may play an early role in the pathogenesis of various AII-mediated vascular diseases. (Supported by the NIH: HL59266.)

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Endothelin-1 mediates the acute narrowing of the arteries in response to decreased blood flow

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Blood vessels are constantly subjected to shear forces that can lead to pathogenesis of vascular disease. The adaptation to shear stress involves acute vasoregulation followed by chronic restructuring of the vessel wall. We hypothesized that acute narrowing of arteries in response to